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The overexpression or amplification of the AIB1 gene is observed in the majority of primary breast tumors, and is also correlated with a large percentage of hereditary and sporadic ovarian carcinomas. The AIB1 protein is a member of the steroid receptor coactivator family of transcriptional regulatory proteins that contain a conserved C-terminal histone acetyltransferase domain, and an N-terminal bHLH-PAS domain. We hypothesize that the HAT and bHLH-PAS domains of AIB1 play critical roles in mediating elevated transcriptional activation levels in AIB1-mediated cancers. The goal of this proposal is to determine the three-dimensional structure of the HAT and bHLH-PAS domains of AIB1 to facilitate the structure-based design of small molecule inhibitors to specifically target AIB1 for therapeutic application. To date, we have prepared several HAT and bHLH-PAS -containing AIB1 recombinants. Each of the HAT-containing domains, either alone or as GST fusion proteins, are unstable suggesting that the HAT domain is not correctly folded and may require association with another protein factor for AIB1-mediated HAT activity. We have overexpressed and purified to homogeneity several N-terminal bHLH-PAS-containing domains that migrate as apparent monomers on gel filtration chromatography. Crystallization efforts to these bHLH-PAS-containing AIB domains are underway to facilitate an X-ray crystal structure determination.

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(5) INTRODUCTION

Gene amplification is a common cause of increased gene expression in human breast cancer (Ingvarsson, 1999; Schwab, 1998). Commonly amplified chromosomal regions located within 17q12, 8q24 and 11q13 encode the proteins Her-2/neu, c-myc and cyclin-D1, respectively, and have been extensively studied both *in vitro* and *in vivo* (Ingvarsson, 1999; Schwab, 1998) In addition to these chromosomal regions, the long arm of chromosome 20 (20q) has received considerable attention since it encodes a gene termed AIB1 (amplified in breast cancer-1) (Guan et al., 1996). The overexpression or amplification of the AIB1 gene is observed in the majority of primary breast tumors (Anzick et al., 1997), and is also correlated with a large percentage of hereditary and sporadic ovarian carcinomas (Tanner et al., 2000). The AIB1 protein is a member of the steroid receptor coactivator family of transcriptional regulatory proteins that contain a conserved C-terminal histone acetyltransferase (HAT) domain, and an N-terminal bHLH-PAS domain (Leo and Chen, 2000). We hypothesize that the HAT and bHLH-PAS domains of AIB1 play critical roles in mediating elevated transcriptional activation levels in AIB1-mediated cancers. The goal of this proposal is to determine the three-dimensional structure of the HAT and bHLH-PAS domains of AIB1 to facilitate the structure-based design of small molecule inhibitors to specifically target AIB1 for therapeutic application.

The specific technical objectives of this proposal are to: (1) Clone for overexpression and purify recombinant bHLH-PAS and HAT domains of AIB1 (tasks 1-4), (2) Characterize the respective functions of the recombinant bHLH-PAS and HAT domains *in vitro* (tasks 5-6), (3) Prepare crystals of a functionally active bHLH-PAS and HAT domain protein construct suitable for structure determination using X-ray crystallography (tasks 7-9), and (4) Determine the X-ray crystal structure of the bHLH-PAS and HAT domains of AIB1 using X-ray crystallography (tasks 10-15). We expect that these studies will provide a scaffold for the design of AIB1-specific inhibitory molecules that will be useful for the treatment of AIB1-mediated breast and ovarian cancer.

(6) BODY

During the first year of the proposal we had made progress towards Technical Objective 1 (tasks 1-4). Specifically, we had cloned for overexpression in bacteria several N-terminal bHLH-PAS and C-terminal HAT domains. We were able to successfully purify to homogeneity, milligram amounts of two bHLH-PAS domains that were suitable for crystallization efforts, 6xHis-AIB1(1-326) and 6xHis-AIB1(1-405). The shortest AIB1 protein construct, 6xHis-AIB1(1-326), contains the complete bHLH, PAS-A and PAS-B domains only, and the longer AIB1 construct, 6xHis-AIB1(1-405), also contained a ~65-residue C-terminal region that is highly conserved among the Steroid Receptor Coactivator proteins. We obtained poor yield of the non-6xHis-tagged versions of these proteins, and were therefore not able to biochemically characterize them.

Towards completing Technical Objective 1, we had also cloned for overexpression in bacteria C-terminal HAT domain containing constructs of AIB1, these protein constructs included residues 975-1412 of AIB1 [AIB1(975-1412)], as an N-terminal 6xHis-tagged and N-terminal GST-tagged fusion proteins. Both protein constructs expressed to high levels in bacteria

but were found exclusively in the inclusion bodies of the bacteria. We concluded that this HAT domain is inherently unstable and may require association with a cofactor protein for protein stability and HAT activity.

Finally, during the first year of the proposal we had also initiated Technical Objective 2 (Task 5) to characterize the multermerization state of the bHLH-PAS domain constructs of AIB1. By using gel filtration chromatography and dynamic light scattering we showed that the bHLH-PAS domain was monomeric.

During the second year of the proposal, we have focused largely on Technical Objective 3 (Task 7), to obtain crystals of the bHLH-PAS domain. To date, we have not yet been successful in this effort. In summary, both the 6xHis-AIB1(1-326) and 6xHis-AIB1(1-405) protein constructs were subjected to extensive crystallization efforts using several different crystallization screens [(Hampton Research Protein Screens I and II and Wizard Screens 1 and 2 (Emerald Biosystems)] at both 18 °C and 4 °C (Task 7). No suitable crystals have yet been obtained, as the majority of the crystallization conditions resulted in the formation of amorphous precipitate.

To address the possibility that the 6xHis fusion tag at the N-terminus of AIB1 may inhibit crystallization, we engineered a TEV protease site between the 6xHis-tag and the bHLH-PAS (1-326) region. This protein construct was purified as we previously reported and the 6xHis-tag was removed using TEV protease. The untagged AIB1(1-326) protein construct was purified by gel filtration chromatography, concentrated to 20 mg/ml and subjected to preliminary crystallization efforts using Hampton Research Protein Screens I and II at 18°C. We have not yet obtained crystals of this protein construct, but additional crystallization efforts are ongoing. We are currently preparing a 6xHis-AIB1(1-405) construct containing a TEV protease site for 6xHis cleavage, and once this is prepared, we will carry out crystallization efforts for the untagged AIB1(1-405) protein construct.

As indicated in last years progress report, we were not able to prepare soluble constructs of AIB1 residues 975-1412 of the C-terminal HAT domain. We hypothesized that the insolubility of this protein construct might be due to the poly-glutamine linker region within residues 1239-1266 of the protein. To get around this potential problem, we cloned for overexpression two subdomains of the C-terminal AIB1 protein, 6xHis-AIB1(975-1239) and 6xHis-AIB1(1266-1412). While we are still in the process of cloning the AIB1(975-1239) protein construct, we successfully cloned the AIB1(1266-1412) construct and also found it to be produced in the insoluble protein fraction when overexpressed in bacteria.

In the final year of the proposal we plan to concentrate our efforts on crystallizing the bHLH-PAS domain of AIB1, and once suitable crystals are obtained, we will initiate structure determination using X-ray crystallography (Technical Objectives 3 and 4). In the event that the untagged AIB1 bHLH-PAS constructs do not form crystals, we will employ the following alternative strategies. First, there has been several recent studies showing that the PAS domain from other proteins bind various adenine dinucleotide analogues such as reduced and/or oxidized forms of NAD or FAD (Bibikov et al., 2000; Gomelsky and Klug, 2002; Rutter et al., 2001).

Based on this observation, we will entertain the possibility that the bHLH-PAS domain of AIB1 may bind adenine dinucleotide analogues, and that an appropriate ligand-bound form of the protein may be more amenable to crystallization. To test this hypothesis, we will first use Isothermal Titration Calorimetry (Cooper and Johnson, 1994; Freire et al., 1990) to assay the ability of the AIB1 bHLH-PAS domains to bind reduced and oxidized forms of NAD and FAD. In addition to identifying ligands that may be useful in crystallizing the AIB1 bHLH-PAS domain, these studies will also address Technical Objective 2 to characterize the function of the AIB1 bHLH-PAS domain. Second, we will subject the AIB1 bHLH-PAS domain protein constructs to limited proteolysis to identify smaller globular domains that may be more emendable for crystallization.

Once suitable crystals are obtained (task8) we will (1) Characterize the diffraction properties of cryogenic crystalline samples of the AIB1 bHLH-PAS (task9), (2) Collect and process a native cryogenic crystallographic data set for crystals of the bHLH-PAS domains of AIB1 (task 10), and (3) Prepare heavy atom/MAD derivatives for crystals and collect X-ray data from the heavy atom – derivatized crystals. This information will be used to determine the three-dimensional structure of the bHLH-PAS domain of AIB1 (task 12).

In the coming year we will also complete the preparation of the C-terminal AIB1 protein construct, AIB1(1266-1412). If we are able to purify this protein to homogeneity, we will assay it for HAT activity (tasks 5-6). If this protein domain is insoluble, we will coexpress it with the AIB1(975-1239) protein construct in the hope that the proteins will fold together (in the absence of the polygutamine linker region) into a soluble heterodimeric species. Once appropriate soluble C-terminal AIB1 HAT domain is prepared we will assay for HAT activity (Technical Objective 2, tasks 5 and 6) and carry out crystallization efforts (tasks 7 and 8) for X-ray structure determination (tasks 9-14).

(7) KEY RESEARCH ACCOMPLISHMENTS

- Cloned and overexpressed in bacteria several different AIB1 protein constructs including the bHLH-PAS domains: 6xHis-AIB1(1-326), 6xHis-AIB1(1-405) with and without the TEV protease sites to remove the 6xHis tags. We also cloned for overexpression in bacteria the HAT domain containing AIB1 construct 6xHis-AIB1(975-1412) and the 6xHis-AIB1(1266-1412) subdomain.
- Purification to homogeneity of the 6xHis-AIB1(1-326), 6xHis-AIB1(1-405) and AIB1(1-326) protein constructs.
- Demonstration that the 6xHis-AIB1(1-326) and 6xHis-AIB1(1-405) protein constructs are monomeric in solution.
- Initiated crystallization efforts for the 6xHis-AIB1(1-326), 6xHis-AIB1(1-405) and AIB1(1-326) protein constructs

(8) REPORTABLE OUTCOMES

None

(9) CONCLUSIONS

Through the second year of the funding period we have successfully carried out tasks 1-5 of the proposal. Specifically, we have overexpressed and purified to homogeneity various AIB1 protein constructs containing the bHLH-PAS domain and crystallization efforts are underway (tasks 7). The preparation of AIB1 protein constructs containing the C-terminal HAT domain has been more problematic, and we continue in our efforts to prepare soluble HAT domain containing protein constructs that may be amenable for crystallization efforts. While we will continue our crystallization efforts of our available AIB1 bHLH-PAS domain constructs (task 7), we will continue to prepare additional bHLH-PAS and HAT domain constructs for crystallization efforts. Once suitable crystals are obtained, we will then employ standard X-ray crystallographic procedures to determine the structure of the bHLH-PAS and HAT domains of AIB1 (tasks 8-12).

Since the AIB1 protein is amplified or overexpressed in the majority of breast tumors, and since the high conservation of the bHLH-PAS and HAT domains implies functional importance, these AIB1 protein domains are highly relevant target for the development of inhibitory compounds that may provide effective therapeutics for the treatment of breast cancer. The structure of the bHLH-PAS and HAT domains will allow us to use structure-based strategies to design "lead" compounds to associate with the protein via its site of action and thereby inhibit the protein function. This lead compound can then be optimized for increased protein binding affinity, specificity and cell delivery properties using a combinatorial chemistry approach (Beeley and Berger, 2000; Kirkpatrick et al., 1999; Leach et al., 2000; Roe et al., 1998). Such compounds may have clinical applications towards the treatment of AIB1-mediated cancers.

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(11) APPENDICES

None